JOURNAL OF BACTERIOLOGY, Jan. 1998, p. 195–200 Vol. 180, No. 2
0021-9193/98/$04.00+0

Catabolite Gene Activator Protein Mutations Affecting Activity of the *araBAD* Promoter

XIN ZHANG† AND ROBERT SCHLEIF*

Department of Biology, Johns Hopkins University, Baltimore, Maryland 21218

Received 23 September 1997/Accepted 4 November 1997

We have studied catabolite gene activator protein (CAP) activation at the *araBAD* promoter, p_{BAD}, in the absence of DNA looping. We ruled out the two most plausible indirect activation mechanisms: CAP-induced folding of upstream DNA back upon RNA polymerase, and CAP-induced stabilization of AraC binding to DNA. Therefore, a direct CAP-RNA polymerase interaction seemed likely. We sought and found CAP mutants defective in transcription activation at p_{BAD} that retained normal DNA binding affinity. Some mutations altered residues in the interval from positions 150 to 164 that includes CAP activating region 1 (AR1), which has been shown to contact RNA polymerase at a number of promoters. Unexpectedly, additional mutations were found that altered residues in the region between positions 46 and 68 and at position 133. This includes the region known as activating region 3 (AR3). Mutations from both groups also affect the *araFGH* and *rhaBAD* promoters.

The presence of multiple activators permits a promoter to respond to multiple environmental cues. The way in which several activators work together is an important question in the study of transcription regulation. The *araBAD* promoter in *Escherichia coli* is regulated by two transcription factors, AraC and catabolite gene activator protein (CAP) (13). The main activator protein, AraC, binds to two direct-repeat half-sites that partially overlap the −35 region of the promoter (8, 32). The second activator, CAP, has a binding site centered at position −93.5 (15). The mechanisms of transcription activation by these two proteins have been studied extensively (5, 10, 32, 38).

AraC protein is composed of a dimerization domain and a DNA binding domain (7). In the absence of arabinose, the dimeric AraC protein binds to the upstream $araO_2$ half-site and the downstream $araI_1$ half-site and forms a DNA loop to repress transcription (9, 26) (Fig. 1). The presence of arabinose induces conformational changes in AraC that lead it to bind to two adjacent half-sites, $araI_1$ and $araI_2$ (24, 26). When bound at $araI_1$ and $araI_2$, AraC helps RNA polymerase to bind to the *araBAD* promoter, p_{BAD}, and also accelerates open-complex formation (38).

CAP is the sole activator at two classes of simple *E. coli* promoters. At class I promoters, where CAP binds at position −61.5 or further upstream, amino acids 156 to 164 of CAP have been shown to be essential for transcription activation (2, 12, 40). This activating region, activating region 1 (AR1), directly contacts the alpha subunit of RNA polymerase (10, 19). At class II promoters, where the CAP binding site is centered at position −41.5, AR1 and amino acids 19, 21, and 101, which constitute activating region 2 (AR2), were both found to interact with the alpha subunit of RNA polymerase (5, 29). Also, amino acids 52 to 58, a region known as activating region 3 (AR3), lie close to the sigma subunit of RNA polymerase (5, 21, 37). In wild-type CAP, AR3 plays little or no role in transcription activation; however, substitution of amino acid 52 can result in increased transcription activation, presumably by creating a new, nonnative interaction between AR3 and sigma (5).

The role of CAP in systems with multiple activators has also been studied. At some promoters where another activator protein is bound between CAP and RNA polymerase, the AR1 region of the upstream CAP has been shown to be involved in transcription activation (6, 23). Apparently, CAP can still contact RNA polymerase even though the two are separated by the third protein. CAP may also act as a structural protein by bending DNA about 90°. It has been argued that this sharp bend may facilitate the binding of upstream DNA to the backside of RNA polymerase (4). CAP-induced bending has been shown to modulate the location of binding of the primary activator MalT to trigger transcription activation in the *malK* promoter (33).

CAP activates *ara* p_{BAD} in at least two ways. Previous studies have established that one role of CAP is to break the loop formed by AraC in the absence of arabinose, and thus CAP "activates" transcription by relieving repression (27). CAP also activates in a loop-independent way (15, 25, 36). When the upstream AraC half-site $araO_2$ was deleted to prevent DNA looping, CAP still substantially activated p_{BAD} (15). Although CAP activation requires the presence of AraC protein, sensitive in vitro studies did not detect any cooperative binding between CAP and AraC (16, 37a), and in a minicircle assay, CAP actually slightly destabilized AraC binding (27).

The loop-independent activation by CAP at *ara* p_{BAD} can be explained if CAP directly contacts RNA polymerase. A possible determinant on CAP for this interaction is AR1, since it has been shown to contact RNA polymerase at a large number of promoters. A previous study with an AR1 mutation, H159L, of CAP however, did not detect any effect at p_{BAD} (37), even though this mutation strongly disrupted the transcription activation of CAP at both class I and class II promoters. This finding left it unclear how CAP stimulates *ara* p_{BAD}.

In this study, we first established that CAP activates the p_{BAD} promoter when DNA looping is impossible. We also showed in in vivo experiments that CAP does not activate transcription by stabilizing AraC binding to the promoter. We

* Corresponding author. Mailing address: Department of Biology, Johns Hopkins University, 3400 N. Charles St., Baltimore, MD 21218. Phone: (410) 516-5207. Fax: (410) 516-5213. E-mail: bio_zrfs@jhuvms.hcf.jhu.edu.

† Present address: Department of Medicine, Brigham and Women's Hospital, Harvard Medical School, Cambridge MA 02115.

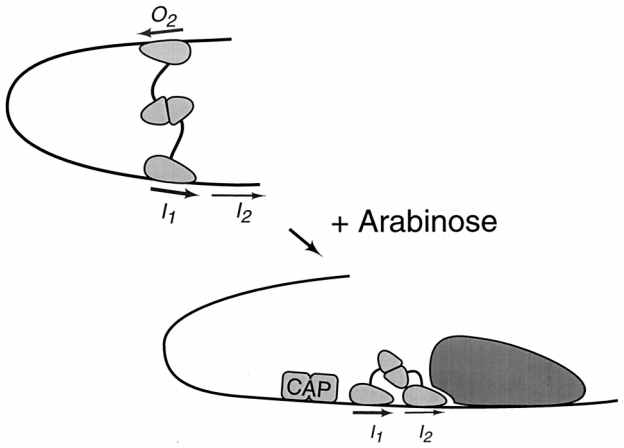

FIG. 1. Regulation of *ara* p_{BAD}. Upon induction of arabinose, AraC releases the upstream *araO*$_2$ half-site and binds to the downstream *araI*$_1$-*araI*$_2$ site. From this position, AraC activates transcription with the aid of the CAP.

then screened for CAP mutants defective in transcription activation and found mutants with amino acid changes in AR1 and in a region overlapping but not coincident with AR3. Our results suggest that CAP directly interacts with RNA polymerase to activate transcription at the *araBAD* promoter.

MATERIALS AND METHODS

General methods, culture media, and conditions. General methods were used as described previously (34). For the β-galactosidase assay and in vivo footprinting, cells were grown in minimal medium, which includes M10 salts, 0.4% glycerol, 10 μg of thiamine per ml, 40 μg of leucine per ml, 0.2% Casamino Acids, and, when added, 0.2% arabinose or L-rhamnose. The β-galactosidase assay was performed as described previously (11). For screening CAP mutants, the indicator plates contained 25.5 mg of antibiotic medium 2 (Difco) per ml, 50 μg of tetrazolium per ml, and 1% lactose.

Strains and plasmids. Strains XE64.2 (Δ*crp39 strA thi*) and XE82 (Δ*crp39 strA thi lacPUV5-O*^{CAP}) were provided by R. H. Ebright (40). To assay promoter activity in an AraC$^-$ background, we used SH321 (Δ*araC-leu*1022, *araB*$^+$*A*$^+$*D*$^+$ Δ*lac*74 *galK Str*^{r}), and for the AraC$^+$ background, we used SH322 [*ara*(*CBAD*)$^+$ *leu* Δ*lac*74 *galK Str*^{r}] (14).

p10 and p10+CAP carry an *ara* p_{BAD} fused to *lacZ*, with p_{BAD} containing the AraC binding site *araI*$_1$-*araI*$_1$*, which is stronger than the wild-type *araI*$_1$-*araI*$_2$ site (32). The sequence of *araI*$_1$*, is 5′-TAGCATTTTTATCCTGA-3′. p10 and p10+CAP were subsequently cloned into the pRS415 plasmid to drive *lacZYA* reporter genes (35), and they are named pXZ36 and pXZ51. pXZ23 is a derivative of p10+CAP with two upstream CAP binding sites separated by 4 bp, centered at positions −93.5 and −108.5, so that the two CAP molecules bind to opposite faces of the DNA.

pXZ59 contains a class I CAP-dependent promoter, *CC+20pmelR*, with its CAP binding site replaced with the *ara* p_{BAD} CAP site (2). pXZ9 carries *CCpmelR*, which is a class II CAP-dependent promoter (2). Both were made by inserting synthesized DNA fragments containing the promoter sequences into pTAP4 plasmids to drive *lacZ* reporter genes (32).

pGBO21B expresses the AraC DNA binding domain (7). pGBO21B was digested with *Pst*I and *Nco*I to delete the AraC gene, which was replaced with the wild-type CAP gene cloned by PCR from the chromosomal template. The new plasmid-expressing CAP protein is named pXZ29. Its sequence was verified by sequencing.

In vivo DMS footprinting. Cells (10 ml) were grown at 37°C in 125-ml flasks to an optical density at 550 nm of 0.8 to 1.0. Dimethyl sulfate (DMS) (10 μl) was directly added to the flask, and the cell culture was shaken for 2 min at 37°C. The culture was transferred to an ice-cold centrifuge tube and centrifuged at 1,000 × *g* for 5 min at 4°C. Plasmid DNA was isolated from the cell pellet and dissolved in 70 μl of distilled H_2O dH_2O plus 10 μl of piperidine. The solution was incubated at 90°C for 30 min, extracted with 1 ml of 1-butanol, and centrifuged at 15,000 × *g* for 15 min. The plasmid pellet was rinsed with 70% ethanol, dried, and resuspended in 15 μl of distilled H_2O. A PCR amplification was carried out in a 10-μl reaction mixture with only one ^{32}P-labeled oligonucleotide primer. For p10, the primer was 5′-AATAGGCGTATCACGAGGCCC-3′. For p10+CAP, the primer was 5′-TTATTTGCACGGCGTCACACTTT-3′. The reaction mixture contained 7 μl of piperidine-treated plasmid, 0.5 U of *Taq* DNA polymerase, 3 ng of ^{32}P-labeled oligonucleotide primer, 10 μM dATP, 10 μM dCTP, 10 μM dTTP, 20 μM dGTP, 10 mM $MgCl_2$, and 50 mM Tris-HCl (pH 9). The cycle parameters were 94°C for 1 min, 60°C for 1 min, and 65°C for 1 min, repeated for 29 cycles. The reaction products were run on a 6% denaturing polyacrylamide sequencing gel.

Random mutagenesis and screening of CAP. The DNA fragment containing the CAP gene was amplified by PCR with primers 5′-TCATCCGCCAAAACA GCC-3′ and 5′-AATTAATCATCCGGCTCG-3′. Mutations were generated due to the high error rate of *Taq* DNA polymerase. The PCR conditions were the same as those described by Zhou et al. (41), and the 100-μl PCR mixture contained 50 mM KCl, 20 mM Tris-HCl (pH 8.0), 1.5 mM $MgCl_2$, 0.01% gelatin, 0.2 mM each deoxynucleoside triphosphate, 200 ng of each primer, 5 U of *Taq* DNA polymerase, and 5 ng of pXZ29 plasmid. The amplified DNA fragments were cut with *Pst*I and *Nco*I and reinserted into pXZ29.

We electroporated competent XE82 cells containing pXZ51 plasmid with plasmids expressing mutagenized CAP and plated them on tetrazolium-lactose plates. As described in Results, only CAP positive-control (pc) mutants for *ara* p_{BAD} yielded Lac$^-$ red colonies. We purified the plasmids from the red colonies and used them to retransform XE82 and XE64.2/pXZ51 to further verify their CAP pc phenotypes. We sequenced 20 plasmids carrying the mutant CAP gene, and 17 of them possessed only a single nucleotide change.

Protein purification and DNA binding. Wild-type and mutant CAP proteins were purified by cyclic AMP (cAMP) affinity chromatography as described previously (39), yielding proteins more than 95% pure as judged from sodium dodecyl sulfate-acrylamide gel electrophoresis. The DNA binding affinities of these CAP proteins were measured by the DNA migration retardation assay (38). The 250-bp DNA fragments used in the assay contain *ara* p_{BAD}, which was amplified from p10+CAP by PCR with the primers 5′-AATAGGCGTATCAC GAGGCCC-3′ and 5′-GATGGGGAGTAAGCTTGGATTCCAATTGCAATC GC-3′. The DNA binding buffer contained 50 mM KCl, 25 mM sodium HEPES (pH 7.4), 2.5 mM $MgCl_2$, 2.5 mM dithioerythritol, 100 μM cAMP, 100 μg of bovine serum albumin per ml, 0.1 mM potassium EDTA, 5% glycerol, and 20 μg of calf thymus DNA per ml. CAP was incubated with 0.03 nM labeled *araBAD* DNA fragments at 37°C for 10 min before the reaction products were subjected to electrophoresis.

RESULTS

CAP can activate the *araBAD* promoter without the involvement of upstream DNA. DNA upstream of the *araBAD* promoter participates in transcription repression by DNA looping (9). The loop is formed when AraC protein binds to the upstream *araO*$_2$ and the downstream *araI*$_1$ half-sites. CAP helps disrupt the loop to unlock the promoter (27). It is not known, however, how CAP activates transcription when the *araO*$_2$ site has been deleted. One possibility is that AraC uses a hidden upstream binding site or nonspecific DNA and still forms a DNA loop that CAP helps to break. To rule out this possibility, we used just the DNA binding domain of AraC (7). This protein fragment does not dimerize and therefore cannot form a DNA loop. The domain can, however, bind to p_{BAD}, with a special *araI*$_1$* half-site replacing *araI*$_2$. *araI*$_1$* binds AraC considerably more tightly than *araI*$_2$ binds AraC (32). As shown in Fig. 2, even in this case, CAP still stimulates the *araBAD* promoter. Apparently this stimulating activity of CAP is independent of AraC-induced DNA looping.

Conceivably, the role of CAP is just to bend DNA so that it will contact the back side of RNA polymerase. This upstream DNA-RNA polymerase interaction could stabilize the transcription complex and enhance the promoter activity. To investigate if this mechanism explains CAP activation at p_{BAD}, we constructed a variation of the *araBAD* promoter. A second CAP site was placed upstream from the first and positioned so that it bends the DNA away from RNA polymerase. Again, as shown in Fig. 2, we did not see any reduction of CAP activation at this promoter. These experiments indicate that the mechanism of CAP activation at *ara* p_{BAD} does not involve merely bending the DNA.

CAP does not affect AraC binding in the unlooped state. CAP does not significantly activate p_{BAD} in the absence of AraC (data not shown). It is conceivable, then, that CAP may just stabilize AraC binding to DNA or force AraC to bend to reach a conformation from which it can activate transcription. Either way, CAP would influence the binding of AraC to DNA.

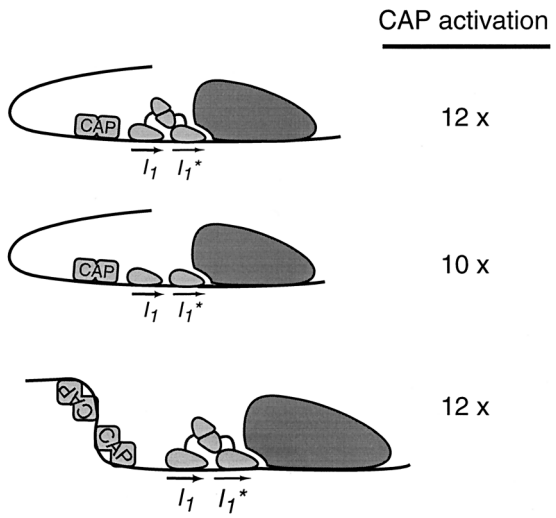

FIG. 2. CAP activation at *ara* p_{BAD} without the aid of upstream DNA. (Top) In the presence of wild-type AraC, CAP can activate *ara* p_{BAD}-I_1-I_1* about 12-fold. (Middle) In the presence of the AraC DNA binding domain, CAP can also activate transcription from *ara* p_{BAD}-I_1-I_1* about 10-fold. (Bottom) At *ara* p_{BAD}-I_1-I_1*, with an additional CAP binding site to bend DNA away from RNA polymerase, CAP can still activate about 12-fold. The promoter activity was measured by the β-galactosidase assay in SH322 cells or in SH321 cells with the AraC DNA binding domain expressed from plasmid pGBO21B. Arabinose was not present. The transcription activation by CAP was calculated by comparing two promoters with or without CAP binding sites.

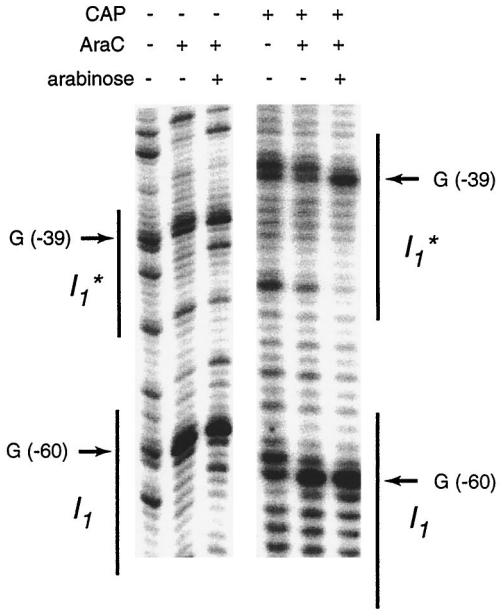

FIG. 3. In vivo DMS footprinting shows that AraC binding is not affected by CAP. The left three lanes show the DMS footprinting of plasmid p10, which has the *ara* p_{BAD}-I_1-I_1* promoter without a CAP binding site. The right three lanes show the footprinting at p10+CAP plasmid, which has the *araBAD*-I_1-I_1* promoter with a CAP site. The footprinting was done in SH321 or SH322 cells.

To assess possible CAP-AraC interactions in vivo, we compared the amount of AraC binding in the presence and absence of CAP. We performed DMS footprinting at *araBAD* promoters with and without the CAP binding site. Neither promoter possessed the $araO_2$ half-site, and thus CAP can stimulate them only directly, not by assisting in loop breaking. If CAP affects the amount of AraC binding, we should be able to detect a difference of AraC footprinting between these two promoters.

In the footprinting assay, cells in the exponential growth phase were treated with DMS. If AraC binds to DNA, two guanines in the AraC binding site become hypersensitive to DMS attack, and the extent of attack correlates well with the occupancy of the site by AraC protein (32). The left three lanes of Fig. 3 show the footprint of the *araBAD* promoter lacking the CAP binding site. When arabinose was added, AraC bound DNA and G(−39) and G(−60) became hypersensitive to DMS. The right three lanes show the footprint of the *araBAD* promoter that possesses the CAP binding site. In both the presence and absence of arabinose, the footprint by AraC was the same as on the promoter without the CAP binding site. AraC binding to DNA was not detectably altered by the presence of CAP. We also assayed the activities of the two promoters in vivo and found that the *araBAD* promoter with the CAP site was about 10 times more active than the one without CAP, even in the absence of arabinose. These results indicate that CAP does not activate transcription by affecting the amount of AraC binding to DNA.

Isolation of *crp* pc mutants for the *araBAD* promoter. Having excluded other reasonable possibilities, we considered the possibility that CAP directly interacts with RNA polymerase through protein-protein interactions. If this were the case, we should be able to isolate mutants of CAP defective in the interaction. The desired mutants should still bind DNA normally, however, and could be called pc mutants.

The wild-type *araBAD* operon is not suitable for the isolation of pc mutants. CAP mutants could disable the arabinose transport operons and lower the arabinose concentration in vivo. This, in turn, would affect induction by AraC and indirectly influence the transcription activity of *ara* p_{BAD}. Another complication is that the assay of wild-type *araBAD* operon activity involves the measurement of arabinose metabolism, which again relies on the arabinose transport operons. DNA looping at wild-type p_{BAD} could also mask the CAP pc phenotype. To avoid these indirect effects, we based our selection on plasmid pXZ51 which carries p_{BAD}-$araI_1$-$araI_1$* fused to *lacZYA*. This variant of the wild-type p_{BAD} promoter possesses a normal basal level and can be activated by CAP to a high level even when AraC is not induced by arabinose (reference 32 and data not shown). This promoter lacks the upstream $araO_2$ half-site, and thus CAP activates transcription independent of DNA looping. Hence, we completely bypassed DNA looping and the arabinose transporter genes to isolate CAP mutants specifically for *ara* p_{BAD}. We further characterized our candidates by examining their behavior at two variant *ara* p_{BAD}-$araI_1$-$araI_1$* promoters, one containing and one lacking a CAP binding site. This latter tests prevents possible confusion from any effects of the CAP mutants on AraC protein expression.

For the isolation of CAP pc mutants effective at the *araBAD* promoter, we used a screen similar to those used previously for the isolation of CAP pc mutants at the *lac* promoter (40). Plasmids expressing a mutagenized *crp* gene were transformed into XE82/pXZ51. This strain carries a chromosomal p_{lacUV5}-O^{CAP} promoter driving *lacZYA*, which can be repressed when CAP binds to a site downstream from the promoter and blocks RNA polymerase. DNA binding mutants of CAP do not block RNA polymerase and therefore do not prevent *lacZYA* expression. Wild-type CAP activates the *ara* p_{BAD} promoter that is fused to *lacZYA* in the pXZ51 plasmid and gives Lac^+ colonies. Only CAP pc mutants, which can bind to DNA but cannot activate *ara* p_{BAD}-*lacZYA*, give Lac^- colonies.

We used PCR to amplify DNA encoding CAP in about 30

TABLE 1. Properties of the CAP mutants with mutations at the *ara* p_{BAD} promoter

CAP mutant[a]	No. of isolates	Activation at *ara* p_{BAD}-I_1-I_1*[b]	Activation at *ara* p_{BAD}-I_1-I_2[c]	Repression at p_{lacUV5}-O^{CAPd}
Wild type		5×	5×	7×
-CAP		1×	1×	1×
Leu-150→Pro	1	1.3×		4×
Ala-156→Gly	1	4×		5×
His-159→Pro	1	1.5×	1×	5×
Gly-162→Asp	3	2×		6×
Gly-162→Ser	2	1.5×		7×
Gln-164→Pro	1	2×		3×
Ser-46→Pro	1	2×		5×
Ile-51→Thr	1	1×	1×	6×
Lys-52→Glu	1	2×	3×	6×
Ile-60→Val	1	2×		6×
Leu-64→Pro	2	1.5×	2×	6×
Asp-68→Gly	1	2×	3×	6×
Asn-133→Asp	1	2×		6×

[a] The first group contains AR1 mutations. The second group has mutations clustered around the AR3 region.
[b] The CAP activation was calculated by comparing *ara* p_{BAD}-I_1-I_1* promoter activity with and without a CAP binding site in the absence of arabinose in XE64.2 cells.
[c] The CAP activation was calculated by comparing *ara* p_{BAD}-I_1-I_2 promoter activity with and without a CAP binding site in the presence of arabinose in XE64.2 cells.
[d] The repression of the p_{lacUV5}-O^{CAP} promoter by CAP was determined in XE82 cells. In the absence and presence of wild-type CAP, the β-galactosidase expression of p_{lacUV5}-O^{CAP} is 40 and 280 U, respectively.

TABLE 2. Effects of CAP pc mutants at CC*pmelR*, *araFGH*, and *rhaBAD* promoters[a]

CAP mutant[b]	Activation of:		
	CCpmelR	*araFGH*	*rhaBAD*
Wild type	30×	11×	27×
Leu-150→Pro	18×	17×	
Ala-156→Gly	23×	1×	
His-159→Pro	4×	1×	2×
Gly-162→Asp	7×	5×	
Gly-162→Ser	7×	1×	2×
Gln-164→Pro	10×	1×	
Ser-46→Pro	10×	6×	
Ile-51→Thr	10×	1×	
Lys-52→Glu	144×	12×	5×
Ile-60→Val	30×	6×	
Leu-64→Pro	15×	3×	2×
Asp-68→Gly	30×	6×	
Asn-133→Asp	35×	7×	

[a] The CAP activation at these promoters was determined in XE64.2 cells by the β-galactosidase assay. CAP mutants were expressed from plasmid pXZ29.
[b] See Table 1, footnote *a*.

independent reactions and inserted the mutagenized DNA into CAP-expressing plasmids. We screened 30,000 colonies, of which 270 were Lac$^-$, and 20 of them yielded Lac$^-$ colonies upon retransformation. As shown in Table 1, these mutants fall into two groups. The first group includes mutants with mutations from residue 150 to 164 of CAP. This includes AR1 of CAP. Of these mutants, those with His-159→Pro, Gly-162→Ser, or Gly-162→Asp have been isolated previously as AR1 mutants (30). The second group of mutants includes those with changes in amino acids 46 to 68 and 133. These amino acids are in or near the antiparallel β-roll region of CAP that contains Lys-52. The amino acids overlap but are not coincident with AR3.

Characterization of the CAP pc mutants. The CAP mutants we isolated repress the p_{lacUV5}-O^{CAP} promoter as well as wild-type CAP (Table 1). This indicates that they are not defective in DNA binding, although it is possible that L150P and Q164P owe their reduced activation effects merely to weakened DNA binding. In vivo analysis shows that all these CAP mutants are defective in activating the *ara* p_{BAD} promoter, in both the presence and absence of arabinose (Table 1). These results show that the CAP mutants we isolated meet the criteria of pc mutants for p_{BAD}.

The first group of our mutants contains those previously found in the AR1 group. The mutants in this group that have been studied previously affected both class I and class II CAP-dependent promoters. Not surprisingly, our mutants were deficient in activating a *lac*-like class I promoter (data not shown) and *CCpmelR* (2), a class II promoter.

The mutants in our second group are moderately reduced in their ability to activate the *lac*-like promoter and the *CCpmelR* promoter. It has been previously shown that mutations in Lys-52 make CAP more active at class II promoters (2). Table 2 shows that our AR3 mutant, K52E, also activates the *CCpmelR* promoter fourfold more than wild-type CAP does.

Since most of our second group of mutants have not been previously isolated, we purified three of them and the wild-type CAP protein for further analysis. Their DNA binding affinity to the *araBAD* promoter was measured by the DNA migration retardation assay. Two mutants bind to DNA as tightly as wild-type CAP does (K_d, 50 and 40 nM for L64P and D68G, respectively; K_d, 50 nM for the wild type) and K52E binds with about a threefold-lower affinity (K_d, 150 nM). We conclude that the DNA binding activities of our mutants are not substantially altered. Figure 4 shows the data for the wild type and the D67G mutant.

Do our pc mutations affect other promoters regulated by AraC or AraC family members? At the *araFGH* promoter, CAP binds at a site adjacent to RNA polymerase and AraC binds to the upstream site (17, 22). At the *rhaBAD* promoter, which is regulated by an AraC family member, RhaR binds immediately adjacent to RNA polymerase and CAP binds upstream from RhaR (11). As Table 2 shows, mutations in AR1 of *crp* affect both promoters. Interestingly, although the second group of CAP mutants, including the mutant with a mutation at Lys-52, have a large effect at the *rhaBAD* promoter, these mutations have much less of an effect on activation of the *araFGH* promoter.

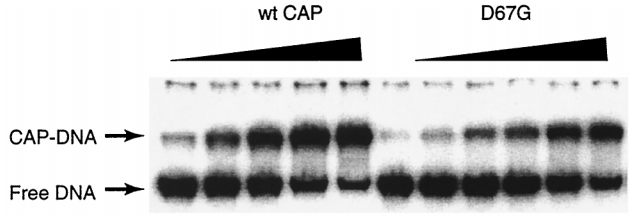

FIG. 4. DNA migration retardation assay of CAP binding to DNA. The concentration of DNA fragments containing the *ara* p_{BAD}-I_1-I_1* promoter was 0.03 nM. The concentrations of wild-type (wt) CAP protein from lanes 1 to 5 were 6.3, 12.5, 25, 50, and 100 nM. The concentrations of CAP mutant D67G from lanes 6 to 11 were 1.3, 2.5, 5, 10, 20, and 40 nM. The occupancy of CAP binding site increased with higher concentrations of CAP, and the equilibrium dissociation constant was calculated as shown in the text.

DISCUSSION

The role of CAP as a coactivator at the *araBAD* promoter has been investigated in this study. We showed that CAP can stimulate transcription at this promoter in a loop-independent way that does not involve altering AraC DNA binding. We have identified two regions of CAP that are important for activation of the *araBAD* promoter and the related *araFGH* and *rhaBAD* promoters. In light of extensive work by others, our results suggest that CAP directly interacts with RNA polymerase at *ara* p_{BAD}, *ara* p_{FGH}, and *rha* p_{BAD}.

At the *araBAD* promoter, the AraC protein binding site is centered at position −52.5 and CAP is centered at position −93.5. How the CAP protein manages to activate transcription from so far away is not clear. AraC protein represses p_{BAD} transcription by forming a DNA loop, and previous experiments have shown that CAP can help open the repression loop and thereby stimulate promoter activity (15, 27), but this does not account for all the CAP activation effect.

An attractive model for CAP activation at *ara* p_{BAD} is that CAP directly interacts with RNA polymerase. This view was cast in doubt, however, when it was reported that the AR1 mutation H159L did not reduce the CAP activation at *ara* p_{BAD} (37). This mutation has been shown to disrupt a large number of promoters where CAP directly interacts with RNA polymerase (10). The confounding study was done with the wild-type *araBAD* operon, and DNA looping was possible (37). Hence, substantial CAP activation could have been seen merely as a result of opening the DNA loop. This may have obscured any direct effects of the AR1 mutation. On the other hand, the result did not exclude the possibility that other regions on CAP were responsible for interaction with RNA polymerase at *ara* p_{BAD}.

We attempted to test the direct-interaction model by isolating CAP mutants defective in transcription activation at *ara* p_{BAD} but not defective in DNA binding. Interestingly, one group of mutants we isolated had altered residues in or near the activating region one of CAP, AR1. Many lines of evidence have suggested that at class I and II promoters, where CAP is the sole activator, AR1 of CAP interacts with the C-terminal domain of the RNA polymerase alpha subunit (5, 10). CAP uses this interaction to recruit RNA polymerase to the promoter and increase its initial binding to DNA. The same mechanism probably applies at the *araBAD* promoter.

If CAP contacts the alpha subunit of RNA polymerase, how does it reach past the interposed AraC protein? If the intervening protein bends the DNA sharply, such an interaction may easily take place. Previous studies have shown that at some promoters with CAP bound around position −91 and another activator at −42, AR1 was also required for CAP to activate transcription (6, 23). Since the C-terminal domain of the alpha subunit is tethered to RNA polymerase through a flexible linker (3, 20), it is conceivable that this domain can reach over another protein to contact the AR1 of the upstream CAP. At *ara* p_{BAD}, AraC-induced DNA bending may also help to move the upstream CAP closer to contact RNA polymerase.

The identification of the second group of *crp* pc mutants at the *araBAD* promoter was unexpected. The mutations clustered around Lys-52, which is contained in AR3 (5). A number of amino acid substitutions at Lys-52, including the one we isolated, can improve CAP activation at class II promoters (2). Also, a previous cross-linking study indicated that Lys-52 is positioned very close to the sigma subunit of RNA polymerase at these promoters (21). It was suggested that mutations at Lys-52 expose a nonnative activating region that can interact with the RNA polymerase sigma subunit to activate transcription at class II promoters (5). It is unclear, however, what the role of AR3 at *ara* p_{BAD} is. Our AR3 mutation K52E reduced CAP activation at p_{BAD}, which could mean that this mutation disrupted an existing activating region of CAP instead of revealing one. Recent studies have indicated that the two alpha subunits of RNA polymerase can each contact upstream activating elements (28). Possibly, one alpha subunit interacts with AR1 and another interacts with AR3 of CAP. It has also been proposed that at class II promoters, functional AR3 interacts with residues in the region of the sigma subunit from positions 590 to 600 (5). Previous studies identified sigma mutations at His-596, which made *ara* p_{BAD} independent of CAP (18), suggesting a sigma-AraC interaction.

A number of the mutations we isolated in the AR3 region are buried inside the protein. These buried amino acids probably cannot contact another protein directly, yet they reduce the CAP activation at p_{BAD}. Perhaps they induce conformational changes in CAP that disrupt the surface-exposed AR1 or AR3 or their accessibilities. We should also point out that residues in this area have been implicated in induction of CAP by cAMP (1). Recently, a second cAMP pocket was found to exist near the DNA binding domain of CAP (31). Its occupancy by cAMP or the effects of its occupancy could be affected by our mutations.

It is not surprising that the CAP AR1 mutants that are defective in transcription activation at *ara* p_{BAD} also affect two related promoters, *ara* p_{FGH} and *rha* p_{BAD}. AR1 of CAP probably directly contacts RNA polymerase at these two promoters. Mutations near AR3 only slightly disturb CAP activation at p_{FGH}, however, as might be expected since this is a class II CAP-dependent promoter that also contains upstream AraC binding sites, and AR3 and the surrounding region in wild-type CAP does not directly participate in transcription activation at class II promoters. At the *araBAD* and *rhaBAD* promoters, however, mutations in the AR3 region strongly reduce CAP activation. These two promoters both have another activator protein positioned between CAP and RNA polymerase, as opposed to class II CAP-dependent promoters, where CAP binds adjacent to RNA polymerase.

Our results indicate that CAP plays a direct role in transcription activation at *ara* p_{BAD}. We have demonstrated that CAP can activate *ara* p_{BAD} without altering DNA looping or AraC binding. A number of CAP pc mutants defective at p_{BAD} were isolated, and they fell into the regions of CAP known to interact with RNA polymerase at many promoters. These lines of evidence suggest that direct CAP-RNA polymerase interactions also occur at *ara* p_{BAD}.

ACKNOWLEDGMENTS

We thank Richard Ebright and Wei Niu for discussions and materials, Steve Busby, and Richard Gourse for comments, and members of our laboratory for ongoing discussions.

This work was supported by National Institutes of Health grant GM18277 to R.F.S.

REFERENCES

1. **Aiba, H., T. Nakamura, H. Mitani, and H. Mori.** 1985. Mutations that alter the allosteric nature of cAMP receptor protein of *Escherichia coli*. EMBO J. **4:**3329–3332.
2. **Bell, A., K. Gaston, R. Williams, K. Chapman, A. Kolb, H. Buc, S. Minchin, J. Williams, and S. Busby.** 1990. Mutations that alter the ability of the *Escherichia coli* cyclic AMP receptor protein to activate transcription. Nucleic Acids Res. **18:**7243–7250.
3. **Blatter, E. E., W. Ross, H. Tang, R. L. Gourse, and R. H. Ebright.** 1994. Domain organization of RNA polymerase alpha subunit: C-terminal 85 amino acids constitute a domain capable of dimerization and DNA binding. Cell **78:**889–896.
4. **Bracco, L., D. Kotlarz, A. Kolb, S. Diekmann, and H. Buc.** 1989. Synthetic

curved DNA sequences can act as transcriptional activators in *Escherichia coli*. EMBO J. **8:**4289–4296.
5. **Busby, S., and R. Ebright.** 1997. Transcription activation at class II CAP-dependent promoters. Mol. Microbiol. **23:**853–859.
6. **Busby, S., D. West, M. Lawes, C. Webster, A. Ishihama, and A. Kolb.** 1994. Transcription activation by the *Escherichia coli* cyclic AMP receptor protein. J. Mol. Biol. **241:**341–352.
7. **Bustos, S. A., and R. F. Schleif.** 1993. Functional domains of the AraC protein. Proc. Natl. Acad. Sci. USA **90:**5638–5642.
8. **Carra, J. H., and R. F. Schleif.** 1993. Variation of half-site organization and DNA looping by AraC protein. EMBO J. **12:**35–44.
9. **Dunn, T. M., S. Hahn, S. Ogden, and R. F. Schleif.** 1984. An operator at −280 base pairs that is required for repression of *araBAD* operon promoter: addition of DNA helical turns between the operator and promoter cyclically hinders repression. Proc. Natl. Acad. Sci. USA **81:**5017–5020.
10. **Ebright, R. H.** 1993. Transcription activation at class I CAP-dependent promoters. Mol. Microbiol. **8:**797–802.
11. **Egan, S. M., and R. F. Schleif.** 1993. A regulatory cascade in the induction of *rhaBAD*. J. Mol. Biol. **234:**87–98.
12. **Eschenlauer, A. C., and W. S. Reznikoff.** 1991. *Escherichia coli* catabolite gene activator protein mutants defective in positive control of *lac* operon transcription. J. Bacteriol. **173:**5024–5029.
13. **Greenblatt, J., and R. Schleif.** 1971. Arabinose C protein: regulation of the arabinose operon *in vitro*. Nat. New Biol. **233:**166–170.
14. **Hahn, S., T. Dunn, and R. Schleif.** 1984. Upstream repression and CRP stimulation of the *Escherichia coli* L-arabinose operon. J. Mol. Biol. **180:**61–72.
15. **Hahn, S., W. Hendrickson, and R. Schleif.** 1986. Transcription of *Escherichia coli ara in vitro*. The cyclic AMP receptor protein requirement for p_{BAD} induction that depends on the presence and orientation of the $araO_2$ site. J. Mol. Biol. **188:**355–367.
16. **Hendrickson, W., and R. F. Schleif.** 1984. Regulation of the *Escherichia coli* L-arabinose operon studied by gel electrophoresis DNA binding assay. J. Mol. Biol. **178:**611–628.
17. **Hendrickson, W., C. Stoner, and R. Schleif.** 1990. Characterization of the *Escherichia coli araFGH* and *araJ* promoters. J. Mol. Biol. **215:**497–510.
18. **Hu, J. C., and C. A. Gross.** 1985. Mutations in the sigma subunit of *E. coli* RNA polymerase which affect positive control of transcription. Mol. Gen. Genet. **199:**7–13.
19. **Igarashi, K., and A. Ishihama.** 1991. Bipartite functional map of the *E. coli* RNA polymerase alpha subunit: involvement of the C-terminal region in transcription activation by cAMP-CRP. Cell **65:**1015–1022.
20. **Jeon, Y., T. Yamazaki, T. Otomo, A. Ishihama, and Y. Kyogoku.** 1997. Flexible linker in the RNA polymerase alpha subunit facilitates the independent motion of the C-terminal activator contact domain. J. Mol. Biol. **267:**953–962.
21. **Jin, R., K. A. Sharif, and J. S. Krakow.** 1995. Evidence for contact between the cyclic AMP receptor and sigma^{70} subunit of *E. coli* RNA polymerase. J. Biol. Chem. **270:**19213–19216.
22. **Johnson, C. M., and R. F. Schleif.** 1995. In vivo induction kinetics of the arabinose promoters in *Escherichia coli*. J. Bacteriol. **177:**3438–3442.
23. **Joung, J. K., L. U. Le, and A. Hochschild.** 1993. Synergistic activation of transcription by *Escherichia coli* cAMP receptor protein. Proc. Natl. Acad. Sci. USA **90:**3083–3087.
24. **Lee, N., C. Francklyn, and E. P. Hamilton.** 1987. Arabinose-induced binding of AraC protein to *araI2* activates the *araBAD* operon promoter. Proc. Natl. Acad. Sci. USA **84:**8814–8818.
25. **Lichenstein, H. S., E. P. Hamilton, and N. Lee.** 1987. Repression and catabolite gene activation in the *araBAD* operon. J. Bacteriol. **169:**811–822.
26. **Lobell, R. B., and R. F. Schleif.** 1990. DNA looping and unlooping by AraC protein. Science **250:**528–532.
27. **Lobell, R. B., and R. F. Schleif.** 1991. AraC-DNA looping: orientation and distance-dependent loop breaking by the cyclic AMP receptor protein. J. Mol. Biol. **218:**45–54.
28. **Murakami, K., M. Kimura, J. T. Owens, C. F. Meares, and A. Ishihama.** 1997. The two alpha subunits of *Escherichia coli* RNA polymerase are asymmetrically arranged and contact different halves of DNA upstream element. Proc. Natl. Acad. Sci. USA **94:**1709–1714.
29. **Niu, W., Y. Kim, G. Tau, T. Heyduk, and R. H. Ebright.** 1996. Transcription activation at class II CAP-dependent promoters: two interactions between CAP and RNA polymerase. Cell **87:**1123–1134.
30. **Niu, W., Y. Zhou, Q. Dong, Y. W. Ebright, and R. H. Ebright.** 1994. Characterization of the activating region of *Escherichia coli* catabolite gene activator protein (CAP). I. Saturation and alanine-scanning mutagenesis. J. Mol. Biol. **243:**595–602.
31. **Passner, J., and T. Steitz.** 1997. The structure of a CAP-DNA complex having two cAMP molecules bound to each monomer. Proc. Natl. Acad. Sci. USA **94:**2843–2847.
32. **Reeder, T., and R. Schleif.** 1993. AraC protein can activate transcription from only one position and when pointed in only one direction. J. Mol. Biol. **231:**205–218.
33. **Richet, E., and L. Sögaard-Andersen.** 1994. CRP induces the repositioning of MalT at the *Escherichia coli malKp* promoter primarily through DNA bending. EMBO J. **13:**4558–4567.
34. **Schleif, R., and P. Wensink.** 1981. Practical methods in molecular biology. Springer-Verlag, New York, N.Y.
35. **Simons, R. W., F. Houman, and N. Kleckner.** 1987. Improved single and multicopy *lac*-based cloning vectors for protein and operon fusions. Gene **53:**85–96.
36. **Stoltzfus, L., and G. Wilcox.** 1989. Effect of mutations in the cyclic AMP receptor protein-binding site on *araBAD* and *araC* expression. J. Bacteriol. **171:**1178–1184.
37. **Williams, R., A. Bell, G. Sims, and S. Busby.** 1991. The role of two surface exposed loops in transcription activation by the *Escherichia coli* CRP and FNR proteins. Nucleic Acids Res. **19:**6705–6712.
37a. **Withey, J., and R. Schleif.** Unpublished data.
38. **Zhang, X., T. Reeder, and R. Schleif.** 1996. Transcription activation parameters at *ara* p_{BAD}. J. Mol. Biol. **258:**14–24.
39. **Zhang, X. P., A. Gunasekera, Y. W. Ebright, and R. H. Ebright.** 1991. Derivatives of CAP having no solvent-accessible cysteine residues, or having a unique solvent-accessible cysteine residue at amino acid 2 of the helix-turn-helix motif. J. Biomol. Struct. Dyn. **9:**463–473.
40. **Zhou, Y., X. Zhang, and R. H. Ebright.** 1993. Identification of the activating region of catabolite gene activator protein (CAP): isolation and characterization of mutants of CAP specifically defective in transcription activation. Proc. Natl. Acad. Sci. USA **90:**6081–6085.
41. **Zhou, Y. H., X. P. Zhang, and R. H. Ebright.** 1991. Random mutagenesis of gene-sized DNA molecules by use of PCR with *Taq* DNA polymerase. Nucleic Acids Res. **19:**6052.